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TITLE: Method for Scavenging Radicals with Urocanic Acid, Derivatives and Analogues

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METHOD FOR SCAVENGING RADICALS WITH UROCANIC ACID, DERIVATIVES AND ANALOGUES

The invention relates to antioxidants or radical scavengers and their reaction products.

Trans-urocanic acid (*trans*-UCA) is a major ultraviolet (UV) absorbing component of the human epidermis. Absorption of UV radiation from the UV-C region (200 - 290 nm) into the UV-A-I region (340 - 400 nm) causes photoisomerization of *trans*-UCA into *cis*-UCA *in vivo* as well as *in vitro* [1-3]. Because of this property, *trans*-UCA has been used as natural sunscreen agent [4]. This use had later been minimized since it became clear that photoproduct *cis*-UCA can mimic some of the effects of UV on immunity, suggesting that this compound is an important mediator of UV-induced immunosuppression [5], however, at the moment it is not clear what the main role of UCA or its mode of action is in the context of immunomodulation. Although experiments *in vivo* supply evidence for the immuno-suppressive potential of *cis*-UCA (8-12), it is remarkable that in a number of cell cultures (*in vitro*) suppression was not found (13-17). Similar levels of *cis*-UCA can be induced by UV-A and UV-B, but nevertheless UV-B is more potent in suppressing contact hypersensitivity than UV-A (18).

The invention provides compounds and compositions for use in methods for scavenging radicals or for modulating the immune response comprising urocanic acid or salts, derivatives, functional equivalents and analogues thereof. Said compounds, compositions and methods as provided by the invention are based on the novel insight that urocanic acid isomers are radical scavengers and serve as natural antioxidants in the body, in particular in skin. UV exposure of the skin causes an increased level of oxidative stress with the inherent formation of reactive (hydroxyl) radicals. It is shown herein that (salts of) urocanic acid isomers or functional equivalents such as imidazole equivalents and

imidazolone derivatives thereof, in particular physiologically (in the body) occurring imidazole compounds for example act as physiological antioxidants capable of efficiently protecting lipid phases of biological membranes and proteinaceous substances in aqueous environments against the action of radicals such as hydroxyl, singlet oxygen or other reactive odd-electron species. These species can be generated from hydrogen peroxide upon UV irradiation, and from hydrogen peroxide in presence of metal ions (e.g. Fe^{2+}), the Fenton reaction. Both types of reaction can occur in the epidermis [6]. Under conditions of oxidative stress, enhanced by exposure to UV [7], it is evident that UCA isomers will encounter the randomly produced hydroxyl radicals *in situ*.

The invention thus provides in one embodiment a method for scavenging radicals in a substance comprising providing said substance with urocanic acid or a functional equivalent thereof, such as a salt or functionally related imidazole compound. Preferably, *trans*-urocanic acid or a functional equivalent thereof is used, being most active or being least immunosuppressive. Using urocanic acid or equivalents thereof as antioxidant or radical scavenger is advantageous over using other antioxidants, such as vitamin E, which are commonly not or only partly soluble in water, whereas urocanic acid or its analogues dissolve easily in aqueous solutions. Especially where said substance comprises a food product or cosmetic product, which are commonly water based, using urocanic acid or its functional equivalent as provided by the invention is advantageous over water insoluble antioxidants. Both isomers are water soluble hydroxyl radical scavengers and can be used in the water phase of numerous emulsions. Furthermore, urocanic acid isomers, being natural components of the body, are essentially non-toxic, which additionally is advantageous when preparing a food product or cosmetic product.

In another or subsequent embodiment, the invention thus provides a method for scavenging radicals in a tissue, for

example subjective to oxidative stress, comprising providing said tissue with urocanic acid, e.g. the invention provides use of urocanic acid or equivalents thereof for the preparation of a pharmaceutical or cosmetic composition, for example for the treatment of oxidative stress, such as for example manifested in wrinkles and other signs of ageing tissue, in particular skin. Oxidative stress in living organisms and their tissues, in particular the oxidation of proteins, has been implicated in the phenomenon of ageing, wrinkling, acute damage of proteins, ischemia reperfusion, atherosclerosis, and many chronic diseases, such as psoriasis, scleroderma, lupus erythematosus, allergic contact dermatitis, vitiligo, lichen planus and graft-versus-host disease, or which treatment the invention now provides a pharmaceutical or cosmetic composition comprising urocanic acid or functional equivalent thereof. Such a composition is advantageously also used for immuno modulatory purposes.

In yet another embodiment, the invention provides use of an oxidation product of urocanic acid or equivalents thereof (such as salts or related imidazole compounds having similar effect) for the preparation of a pharmaceutical composition, in particular wherein said product is an photo-oxidation product. Herein is used the novel insight that as a consequence of radical scavenging, epidermal UCA isomers are converted by reactive oxygen species (ROS) into oxidation products with biological i.e. immunomodulating effects. In contrast to the photoisomerization of UCA, not much attention has as yet been given to the oxidation of UCA. In particular not to the reaction of UCA isomers with the very reactive hydroxyl radicals. Hydroxyl radicals can be generated from hydrogen peroxide upon UV irradiation, and from hydrogen peroxide in contact with reduced metal ions, e.g. ferrous (Fe^{2+}) ions. Both types of reaction can occur in the epidermis (6).

Under conditions of oxidative stress, enhanced by exposure to UV (7), it is evident that UCA isomers will

encounter the randomly produced hydroxyl radicals. We now provide the insight that it is in general not *cis*-urocanic acid *per se* that provides modulation or repression of immune responses, but oxidation products of urocanic acid, that for example have arisen after ultraviolet light (UV) exposure of for example skin. Herein, urocanic acid scavenges radicals created by UV exposure, is thereby oxidised to for example imidazole containing urocanic acid derivatives, such as imidazole-4-carboxyaldehyde, imidazole-4-acetic acid or imidazole-4-carboxylic acid, which subsequently modulate, suppress or mitigate a mounting immune response of the body to the UV induced tissue damage.

By providing insight into this natural mechanism, we provide insight in immune modulating mechanisms that are at work to keep (overly strong) immune responses, for example directed at UV exposure at bay. The invention thus provides use of a pharmaceutical composition comprising an oxidation product of urocanic acid for modulating immune responses against various stimuli, thereby mimicking a, previously unknown, natural action of said product. Herewith the invention provides a method to modulate an immune response of an animal, for example a human being, comprising treating said animal with a pharmaceutical composition comprising an oxidation product of urocanic acid, for example wherein said product is an imidazole such as imidazole-4-carboxyaldehyde, imidazole-4-acetic acid or imidazole-4-carboxylic acid or an imidazolon derivative of urocanic acid such as 3-(4-imidazolon-2yl)-acrylic acid and 3-(4-imidazolon-5-yl) acrylic acid. In particular the invention provides the use of one or more UCA photo-oxidation products as immuno modulator in various skin diseases, such as psoriasis or dermatitis. Furthermore, the invention provides a pharmaceutical composition comprising urocanic acid or functional equivalent thereof for its radical scavenging properties, whereby said composition is additionally used as immuno modulator.

optionally already comprising oxidation products having immune modulatory function.

The invention is further explained in the detailed description without limiting the invention thereto.

Detailed description

Trans-UCA, *cis*-UCA, related imidazoles and non-imidazole compounds were tested with regard to their ability to compete with deoxyribose to scavenge hydroxyl radicals. On exposure to hydroxyl radicals deoxyribose is degraded into malondialdehyde, which reacts with thiobarbituric acid to form a pink chromogen. Powerful hydroxyl-radical scavengers will compete with deoxyribose, resulting in a reduced amount of malondialdehyde [22]. Ten compounds, UCA, UCA analogues, alanine and uric acid (Fig.1) were tested on their ability to scavenge hydroxyl radicals.

Method: the deoxyribose (dR) degradation test. The test was analogous to an earlier described method [22]. Briefly, the reactions were performed in 5 mL screw cap glass tubes in a final volume of 1.0 mL sodium phosphate buffer (50 mM; pH 7.2), containing 3.0 mM 2 deoxy-D-ribose, 0.5 mM hydrogen peroxide and one of the test compounds at graded concentrations. The reaction was started by the addition of premixed disodium EDTA and ferrous iron solution (final concentrations 0.5 mM and 0.2 mM, respectively). The mixture was left for 15 minutes at room temperature. After addition of 1.0 mL 1 % thiobarbituric acid in 50 mM NaOH and 0.75 mL 2.8 % trichloroacetic acid, the tubes were heated for 20 minutes in a boiling water bath. The pink color was read at 532 nm and reciprocal absorption values were plotted against the concentration of the test compound after subtraction of appropriate blanks. A series of six duplicate determinations from test compound dilutions was employed to construct a graph slope for the calculation of a rate constant value. The mean, SD, number of rate constants and the percentage of

inhibition of deoxyribose degradation, calculated for each test compound, are listed. Results. All second-order rate constants for reaction with hydroxyl radicals and, in addition, the percentage inhibition of deoxyribose degradation with equimolar concentrations (3 mM) of scavenger are summarized in Table 1. A typical graph with slopes to derive rate constants from is shown in Fig. 2 for both UCA isomers. *Trans*-UCA and *cis*-UCA are substantially more powerful in scavenging hydroxyl radicals (8.0 and 7.1 x 10^9 $M^{-1}.s^{-1}$, respectively), than the other 4-(5-)-substituted imidazoles, including L-histidine (2.6 x 10^9 $M^{-1}.s^{-1}$). L-histidine, the precursor of UCA, was included as a known moderate scavenger [22-24] with structural similarities to UCA. L-alanine was used as a known poor scavenger [22]. *Trans*-FAA was tested as a non-imidazole acrylic acid derivative, having a furan ring instead. This substitution yielded a very poor scavenging ability.

Other 4-(5-) substituted imidazole analogues, dihydrourocanic acid or 3-(imidazol-4-yl)-propionic acid and imidazole-4-acetic acid, showed moderate scavenging ability, comparable to histidine. Unsubstituted imidazole and its 2-methyl derivative appeared to be stronger scavengers than the UCA isomers. The well-known hydroxyl radical scavenger uric acid showed an excellent abilitiy (27.8 x 10^9 $M^{-1}.s^{-1}$).

Trans-UCA and *cis*-UCA, two epidermal compounds, are good hydroxyl radical scavengers; their ability is less than that of uric acid, but larger than that of the other 4-(5-) substituted imidazoles, e.g. histidine.

Trans-UCA and *cis*-UCA are herein recognized as good hydroxyl radical scavengers. Both isomers occur in substantial concentrations in the epidermis, the latter in the UV-exposed skin. There is strong evidence for the occurrence of hydroxyl radicals in the epidermis, especially upon UV irradiation [7]. Normal human skin contains approximately 200 µM iron [26,27], predominantly complexed

to ferritin. The release of free ferrous ions by UV irradiation [28] and the presence of hydrogen peroxide [29,30] are prerequisites for the generation of hydroxyl radicals. Other reports indicate the UV-induced presence of hydroxyl radicals indirectly since their effects on epidermal constituents could be neutralized with antioxidants [31, 32].

UCA is an imidazole compound and several other imidazole derivatives have already been shown to be good hydroxyl radical scavengers, e.g. histidine [22-24], histamine [33], histidine containing dipeptides [24,34], cimetidine and other histamine (H_2) receptor antagonists [35]. This study reveals that several other imidazoles show similar properties (Table 1). Hydroxyl radicals can react with the imidazole ring to form imidazolone derivatives. Their formation has led to the proposal to use the imidazolones of histidine and histamine as markers for oxidative stress [23,33]. The importance of the imidazole ring in UCA molecules was also demonstrated in our experiments. The poor scavenging ability of trans-FAA, having a furan ring instead, was a remarkable contrast. Furthermore, the presence of the acrylic acid moiety in UCA molecules conjugated with the imidazole ring may account for its increased scavenging ability towards hydroxyl radicals as compared to the other 4-(5-) substituted imidazoles. Unsubstituted imidazole and its 2-methyl derivative are stronger hydroxyl radical scavengers, accentuating that the presence of an imidazole ring is a prerequisite for sufficient hydroxyl radical scavenging ability. However, these compounds do not occur physiologically and are harmful (LD_{50} oral rat 220 mg/kg for imidazole and 1500 mg/kg for 2-methylimidazole).

Trans-UCA and *cis*-UCA do occur physiologically, mainly in the epidermis, with relatively high concentrations. Our findings point to a new physiological role for the UCA isomers, besides the suggested roles of trans-UCA as natural sunscreen agent and *cis*-UCA as immunosuppressant. *Trans*-UCA

and *cis*-UCA may be major epidermal hydroxyl radical scavengers, providing a new view on the antioxidant status of the skin. The findings that 1. UCA isomers are good hydroxyl radical scavengers, though not as strong as uric acid, and that 2. the UCA isomers already occupy relatively high concentrations in the skin, create possibilities to apply the UCA isomers as non-toxic antioxidant additives in food and cosmetics in relatively high concentrations. *Trans*-UCA (commercially available) should be preferred, because *cis*-UCA may exert immunosuppressive effects.

In contrast to the photoisomerization of UCA, not much attention has as yet been given to the oxidation of UCA. In particular, the reaction of UCA isomers with the very reactive hydroxyl radicals should be explored. Hydroxyl radicals can be generated from hydrogen peroxide upon UV irradiation, and from hydrogen peroxide in contact with reduced metal ions, e.g. ferrous (Fe^{++}) ions. UV-A irradiation of *trans*-UCA or *cis*-UCA with hydrogen peroxide only results in UCA photoisomerization and not in UCA photooxidation. The lack of correlation between UV-A-induced *cis*-UCA formation and immunosuppression (18) may be another indication for a role of UCA-oxidation products in skin immunology. These compounds can either be formed in the presence of hydrogen peroxide upon UV-B irradiation or by a Fenton reaction; both reaction types leading to comparable sets of oxidation products as determined by chromatographic patterns. The common oxidizing species of both reaction types is most likely the hydroxyl radical. Starting the oxidation with *trans*-UCA or with *cis*-UCA yielded similar chromatographic patterns. In relation with hydroxyl radical scavenging of the UCA isomers, it should be noted that UCA isomers may as well interfere with UV-induced immunosuppression through scavenging of radical species. The presence of the acrylic acid moiety in UCA molecules conjugated with the imidazole ring may account for its

increased scavenging ability towards hydroxyl radicals as compared to non-conjugated imidazoles, such as histidine and histamine. It may also account for the diversity of the formed oxidation products.

Materials and methods

High Performance Liquid Chromatography (HPLC)

Trans-UCA and *cis*-UCA were separated from each other and from several UCA oxidation products on a 4.6 x 250 mm Alltima C_{18} and a Luna C_{18} reversed-phase column (Alltech, Deerfield, Il and Phenomenex, Torrence, CA, resp.) with a flow of 0.8 mL/min, delivered by P-3500 HPLC-pumps (Pharmacia, Uppsala, Sweden). Samples of 20 to 200 µL were injected by a Promis II autosampler (Spark Holland, Emmen, The Netherlands) and chromatographic data were recorded on an SP 4270 integrator (Spectra Physics, San Jose, CA). Peak area data from samples were only processed under identical HPLC circumstances. A UV-detector (Applied Biosystems, model 759A, Foster City, CA) was set for 226 nm detection. Isocratic elution was performed with 10 mM ammonium formate buffer, containing 0.2 - 0.8 mM tetrabutylammonium (TBA) formate and 1 % acetonitrile (pH 7.2). Collected fractions were acidified with formic acid up to a final concentration of 100 mM and passed through C_{18} solid phase extraction columns (JT Baker, Deventer, The Netherlands) in order to remove TBA.

Photooxidation

A 1-cm quartz cuvette, filled with 1.4 mL sample, was placed in the parallel beam of a filtered 1000 W xenon arc lamp (Oriel, Stratford, CT). The samples were magnetically stirred during irradiation. To minimize infrared (heat) and visible radiation, the beam was passed through a water filter (7 cm), reflected by a dichroic mirror and filtered through a 1-mm UG11 filter. Short wave cut off was achieved by passing the beam through WG280, WG305 or WG335 filters with 3 mm thickness each (Schott-Jena, Mainz, Germany). Xenon lamp emission filtered through WG280 included UV-C, UV-B and UV-A; through WG305 UV-B and UV-A and through WG335 only UV-A was included. Two narrow bands in the UV-B and UV-A spectral regions were selected to monitor the xenon-arc emission. The probe of a calibrated EG&G 550 radiometer (Salem, MA, USA) was equipped with a neutral density filter and narrow band filter type UV-M-IL (Schott-Jena) with a transmission maximum of 21 % at 303 nm and a half-width of 11.5 nm to monitor UV-B or with a type UV-P1L (Schott-Jena) with a transmission maximum of 46 % at 363 nm and a half-width of 7.7 nm to monitor UV-A. Transmission spectra of the optical filters were checked on a Perkin Elmer Lambda 40 UV/VIS spectrometer (Norwalk, CT, USA).

Additional irradiations were performed with fluorescent tubes TL12, used as a UV-B source, and TL10R, used as a UV-A source (Philips, Eindhoven, The Netherlands), on samples that were magnetically stirred in small Petri dishes. The UV-B output was measured with an IL 443 phototherapy radiometer, fitted with a SEE 1240 silicon detector probe and the UV-A output with an IL 442A phototherapy radiometer with a SEE 115 detector probe (International Light, Newburyport, MA, USA).

Fenton oxidation.

UCA isomers (10 or 40 μM) were oxidized with a hydroxyl-radical- generating system that consisted of various concentrations of ferrous ions (10 - 500 μM) and a fixed hydrogen peroxide concentration of 500 μM (the Fenton reagent), either in a sodium phosphate (10 or 20 mM) medium of pH 7.2, or in ultrapure water. In addition, two hydroxyl-radical-generating systems with copper ions (Cu^{2+}) were used, consisting of 50 μM Cu^{2+} with either 500 μM hydrogen peroxide or 5 mM ascorbic acid.

Synthesis of reference compound imidazole-4-carboxaldehyde (4-formylimidazole)

4-(Hydroxymethyl)imidazole-HCl (4 mmol) was stirred together with sodium bicarbonate (6 mmol) in 4 ml methanol for 1 hour at room temperature. The methanol was evaporated and the residue was extracted with a chloroform/methanol 1:1. After centrifugation at 3500 rpm for 5 minutes the supernatant was evaporated and the residue was taken up in 20 ml hot dioxane. 4.4 g manganese dioxide (activated; for synthesis) was added, followed by a reflux reaction for 2 hours. Manganese dioxide was removed by filtration and the filtrate was evaporated. Crystallization was carried out in methanol. The yield was 95 mg of fine off-white crystals, 25 % of maximum yield. The melting range was 168 - 169° C : 173 - 175° C). Melting range of starting material was 108 - 111° C and of the oxidation product imidazole-4-carboxylic acid 294 - 295° C. UV (water) λ_{max} (log ε) 257 nm (3.85).

Results

UCA isomers and photooxidation

The O-O bond of hydrogen peroxide can be cleaved by UV radiation to yield hydroxyl radicals. Because both UCA isomers could effectively scavenge hydroxyl radicals, it is to be expected that UCA will be degraded and/or converted into oxidation products. The ability of simulated solar UV radiation to convert *trans*-UCA in the presence of hydrogen peroxide into photooxidation products was tested *in vitro* and analyzed by reversed-phase HPLC analysis. Hydrogen peroxide eluted close to void volume and *trans*-UCA and *cis*-UCA eluted with markedly different elution times of 20 and 64 min (Fig. 3a-d). The unirradiated control sample did not show any interaction between *trans*-UCA and hydrogen peroxide (Fig. 3a). Exposing 80 µM *trans*-UCA in the absence of hydrogen peroxide at pH 7.2 to WG280-filtered xenon-arc emission (including UV-C and UV-B) resulted only in the formation of *cis*-UCA *via* the process of photoisomerization (Fig. 3b). However, when *trans*-UCA was irradiated in the presence of 500 µM hydrogen peroxide under identical conditions, many additional peaks appeared in the chromatograms and both *trans*-UCA and *cis*-UCA peaks were strongly reduced (Fig. 3c), indicating a certain photochemical conversion or breakdown. Eight main photooxidation products were recognized as new peaks based on retention times and were assigned in the chromatogram (Fig. 3c).

In contrast, when exposures were performed with simulated solar radiation from which both UV-C and UV-B were blocked out by a WG335 filter, virtually no photo-oxidation products were found (Fig. 3d). Only UCA photoisomerization was apparent, which is in accordance with earlier reports (2, 3). The ratio of *trans*-UCA to *cis*-UCA photoisomerization was not affected by the degree of photooxidative breakdown. Blocking out UV-C by the use of the WG305 filter showed intermediate results (Table 2). This irradiation condition has the closest simulation with the spectral UV distribution

of terrestrial solar radiation produced by an overhead sun on a bright day. Tests with the fluorescent lamps TL 12 (UV-B and UV-A; some UV-C) and TL10R (UV-A) confirmed th above findings that UV-B and UV-C have photo-oxidative ability. Although the UV-A dose of the fluorescent lamp was much higher than that of UV-B, the yield of UCA photo-oxidation products was much lower with UV-A (Table 2). The formation of photo-oxidation products was quantified by summing the eight major peak areas (in arbitrary units; peaks A - H). The degree of photo-oxidative breakdown, the yield of photo-oxidation products and the degree of UCA photoisomerization under different irradiation conditions were summarized in Table 2. Taking the various emissions of these UV sources into account, the photo-oxidative ability of UV radiation became substantial with wavelengths shorter than approximately 320 nm. Experiments with *cis*-UCA yielded similar results, except that *cis*-UCA/*trans*-UCA ratios were increased in this series (data not shown).

UCA isomers and Fenton oxidation

In the next series of experiments we studied the Fenton oxidation of UCA, representing another natural oxidation process. *Trans*-UCA and *cis*-UCA isomers were Fenton oxidized by ferrous ions (Fe^{2+}) and hydrogen peroxide at physiological concentrations. The initial hydrogen peroxide concentration was 500 μM and the ferrous ion concentration was varied from 0 to 500 μM. In all Fenton oxidation reactions the degree of UCA-isomer breakdown was calculated from their reduced peak areas. The oxidation reaction must have been completed within 2 minutes for all reaction conditions, because no further breakdown was observed after prolonged incubation. Hydrogen peroxide without Fe^{2+} had no effect on the UCA isomers at all; however, Fe^{2+} without hydrogen peroxide resulted in a slow breakdown of UCA isomers after prolonged incubation (data not shown).

The sequence order of addition of the two Fenton reagents did not markedly affect the UCA breakdown and yield of oxidation products, except at a low UCA concentration of 10 μM. When Fe^{2+} was added after hydrogen peroxide, a larger breakdown and a smaller yield of Fenton-oxidation products were observed, whereas the reversed-sequence order gave opposite results (data not shown).

When the Fenton reaction was performed in water instead of phosphate buffer, the oxidative breakdown of *trans*-UCA was enhanced irrespective of the UCA concentration. The turbidity seen in reactions performed in phoshate buffer (10 mM) with high Fe^{2+} concentration (> 100 μM) was probably due to the formation of insoluble iron phosphate, thereby reducing the free availability of Fe^{2+}. Table 3 summarizes the difference between water and phosphate medium for *trans*-UCA at an initial concentration of 40 μM with respect to its breakdown and the formation of Fenton-oxidation products. Similarly to the photo-oxidation experiments, the peak areas of the 8 major oxidation products

were summed. Comparable results were obtained with *cis*-UCA (data not shown), which finding is in accordance with the comparable rate constants of *trans*-UCA and *cis*-UCA in the deoxyribose degradation experiment (Table 1). A close resemblance was observed between the chromatographic patterns of UCA Fenton oxidation products (not shown) and those of UCA photo-oxidation products. Three of them has been identified (vide infra).

When two other hydroxyl-radical-generating systems based on copper ions (Cu^{2+}) were investigated with *trans*-UCA, the combination of Cu^{2+} (50 µM) and ascorbic acid (5 mM) without hydrogen peroxide caused an almost complete breakdown of *trans*-UCA (3 % left), whereas the system with Cu^{2+} (50 µM) and hydrogen peroxide (500 µM) showed little effect (88 % *trans*-UCA left). Evaluation of the data was difficult with the ascorbate system, because several interfering peaks had occurred in the chromatograms, which were probably derived from ascorbic acid and its oxidation products. Both sytems are considered to be of minor importance for the situation *in vivo*, but these results indicate similarities in oxidative behaviour of the UCA isomers, independent of the nature of the hydroxyl-radical-generating system.

UCA isomers and Fenton oxidation.

In another series of experiments we studied the Fenton oxidation of UCA, representing another natural oxidation process. The initial hydrogen peroxide concentration was 500 µM in all experiments and the ferrous ion concentration was varied from 0 to 400 µM. Four sets of conditions were compared: 1. Fe^{2+} in phosphate buffer pH 7.2 , 2. Fe^{2+} in phosphate buffer plus EDTA , 3. Fe^{2+} without buffer with a initial pH of 5.5 - 5.3 and 4. Cu^{2+} in phosphate buffer plus ascorbate. The degree of breakdown was similar for both UCA isomers. Table 3 shows oxidative breakdown of trans-UCA with

hydrogen peroxide in increasing order: condition 1 < 2 < 4 < 3.

The addition of Fe^{2+} at final concentrations of 100 - 400 µM in phosphate buffer caused a turbid solution of insoluble iron phosphate. Under this condition the smallest degree of breakdown was established. A limited availability of free Fe^{2+} is assumed to reduce the oxidative breakdown of UCA. At the other hand, complexation of Fe^{2+} to EDTA did not cause a turbid reaction mixture and a larger breakdown was established (Table 3). The largest breakdown was seen in the absence of phosphate buffer, with a less defined pH value of 5.5 to 5.3 , dependent on the UCA concentration (40, 100 or 250 µM). At the start of the Fenton reaction in the unbuffered medium, there was a rapid fall of the pH value from 5.1 to 3.4, with initial concentrations of trans-UCA, hydrogen peroxide and ferrous ions of 250, 500 and 400 µM, respectively. We attribute this effect to the unbuffered liberation of relatively strong acids, such as glyoxylic acid (GLX). Similar results of breakdown, though slightly less pronounced, were obtained with *cis* UCA (Table 4). This finding is in accordance with the comparable second order rate constants of *trans*-UCA and *cis*-UCA for hydroxyl radical scavenging (8). Hydrogen peroxide without Fe^{2+} had no effect on the UCA isomers at all; however, Fe^{2+} without hydrogen peroxide resulted in a partial breakdown of the UCA isomers upon prolonged incubation of one day (data not shown).

The primary oxidation products formed are ImCHO and GLX. Additional experiments in which ImCHO was used as starting material, a yield of virtually 100 % ImCOOH was obtained after Fenton- or photooxidation. In UCA samples that were highly oxidized (containing < 4 % UCA) ImCOOH was the major 226 nm absorbing compound, while ImCHO concentration was largely reduced. An additional experiment demonstrated that under this oxidative condition the aldehyde (ImCHO) was oxidized to the carboxylic acid (ImCOOH). GLX was analyzed in lower amounts than ImCHO in all cases studied (Table 3),

except for the Fenton oxidation of 40 μM UCA (Table 4, section 3.1 and 3.4). Trans-UCA and cis-UCA in relatively high concentration of 250 μM were broken down for 78 % and 75 %, respectively, by the unbuffered Fenton oxidation system. Table 4 section 3 also shows that the yield of oxidation products was proportional with the initial UCA concentration. Remarkably, the yield of ImCHO from cis-UCA was substantially larger than from trans-UCA. In the phosphate buffered Fenton system a comparable breakdown and a comparable yield of oxidation products was recorded, irrespective of the initial UCA concentration range from 40 to 250 μM (Table 4, section 1, only results of 40 μM are shown). In the presence of EDTA, a larger breakdown and a higher yield of oxidation products (in particular ImCHO) resulted (Table 4, section 2). This yield was raised as higher initial UCA concentrations were used. In the unbufffered system, the highest degree of breakdown of all tested systems was recorded. The oxidation product yield was the largest of all systems when the initial UCA concentration was high (250 μM) (Table 4, section 3).

When another hydroxyl-radical-generating system, based on copper ions (Cu^{2+}) was investigated, the combination of Cu^{2+} / ascorbic acid / hydrogen peroxide caused a large breakdown of *trans*-UCA (Table 3) and a moderate yield of UCA oxidation products, in favor of ImCOOH. Without ascorbic acid, the system with Cu^{2+} (50 μM) and hydrogen peroxide (500 μM) showed little breakdown (88 % *trans*-UCA left; data not shown). For the situation *in vivo*, one must remember that the epidermal copper content is lower than iron (29).

2.3.4. UCA compared in Fenton and photooxidation

A close resemblance was observed between the chromatographic patterns of UCA Fenton oxidation products and those of UCA photooxidation products (Fig.5). Also under photooxidation an oxidation inhibiting effect was seen in phosphate of pH 7.2, whereas the yield of oxidation products was in favor of ImCHO

(Table 4, section 4 versus 5). In photo- oxidation, the breakdown of cis-UCA was substantially decreas d in comparison with the trans isomer (Table 4, section 4-6). In Fenton oxidation, this effect was less pronounced. The data of Table 4 were given for air saturated solutions. Argon-purging of the solutions, prior to Fenton - or photooxidation, enhanced UCA breakdown as well as the yield of oxidation products, both by a factor 2 to 3. Heating (to 37° C) of argon-purged solutions slightly enhanced the yield of ImCHO.

The data of Table 4 indicate a discrepancy ('gap') between micromoles of UCA isomer broken down and micromoles of oxidation products formed. The smallest 'gap', though still 52 %, was found after the oxidation of cis-UCA in the unbuffered system (section 3). Thin layer chromatography (TLC) gave more insight in the 'gap' products, that were not seen in reversed phase chromatography, using UV detection or fluorescence detection. TLC carried out on silica with the eluent isopropanol / ammonia 25 % (4 : 1) showed an array of elutable, partly overlapping fluorescent spots and a fluorescent spot at the start position (data not shown). However, the initial weight of trans-UCA, introduced in a photooxidation experiment with extensive UCA breakdown (< 4 % of each UCA isomer left over), was not lowered much (~ 14 %) after severe photooxidation. This finding indicated a predominant formation of non-volatile, solid material in stead of gaseous compounds, such as CO_2 and water. The TLC pattern and the weighing experiment points to a possible hydroxyl radical initiated chain reaction of UCA, resulting in the formation of substances that may fill the above mentioned gap. These substances may not be fully detected under the chromatographic conditions used for the simultaneous determination of the UCA isomers, ImCHO and ImCOOH.

2.3.5. Inhibition of contact hypersensitivity.

The inhibitory effects of the UCA oxidation products are illustrated in Fig. 5. Maximum ear swelling response was normalized to 100 %. The largest reduction was obtained with the residue of severely photooxidized UCA (PO mix III), containing less than 4 % residual *cis*-UCA. It resulted in only 19 % ear swelling (81 % reduction of swelling). Even a tenfold dilution of that mix (0.2 g/l) reduced the ear swelling markedly (29 % ear swelling), which is of similar level as the effect of *cis*-UCA in a concentration of 1 g/l (31 % ear swelling). Another remarkable effect was obtained by mixing the three identified imidazoles. When we tested one of the imidazoles alone (1 g/l), only a moderate effect was seen, however, when tested mixed together (1 g/l, each imidazole 0.33 g/l), a synergistic effect was observed (26 % ear swelling). Glyoxylic acid and oxalic acid, as ammonium salts, did not exhibit significant inhibition of CHS.

UCA photo-oxidation on a preparative scale

Concentrations of *trans*-UCA and hydrogen peroxide were largely increased, as was the UV exposure, to obtain larger amounts of UCA photo-oxidation products as collected fractions from the reversed phase column for further analysis. A typical chromatogram is shown in Fig. 4. Four fractions, designated as R_t 8, R_t 10, R_t 14, R_t 17, were finally selected for identification (peak A, 1-3 in Fig.4). Prior to analysis, tetrabutylammonium was removed by solid phase extraction on C_{18} silica.

Identification

R_t 8 was identified as imidazole-4-carboxaldehyde (ImCHO). Its UV-spectrum was identical to the synthesized (see below) reference compound with an absorption maximum of 257 nm. Co-injection of R_t 8 with synthesized imidazole-4-carboxaldehyde resulted in a single chromatographic peak with

a retention time of 8.13 minutes. Further evidence is to be collected (peak A in Fig.4). The amount of ImCHO in the photooxidized UCA sample was gradually reduced upon storage at -20° C.

R_t 10 was identified as imidazole-4-acetic acid. Its UV-spectrum was identical with an absorption maximum of 213 nm. Mass spectrum was obtained with electrospray technique and the dry sample was treated with methanol/HCl and n-butanol/HCl before analysis. A peak at mass 140 was obtained after methylation and at mass 183 after butylation. Consequently, the mass of the original compound was 126. Co-injection of R_t 10 with commercially available imidazole-4-acetic acid resulted in a single chromatographic peak with a retention time of 8.98 minutes (peak 1 in Fig.4).

R_t 14 was identified as imidazole-4-carboxylic acid (ImCOOH). Its UV-spectrum was identical to the commercially obtained reference compound with an absorption maximum of 226 nm. Proton resonance (1H-NMR) analysis was done in D_2O, showing imidazolic protons in a ratio 1:1 with shifts of 7.76 and 7.53 ppm. Mass spectrum was obtained with electrospray technique and the dry sample was treated with methanol/HCl and n-butanol/HCl before analysis. A peak at mass 126 was obtained after methylation and at mass 169 after butylation. Consequently, the mass of the original compound was 112. Co-injection of R_t 14 with commercially available ImCOOH resulted in a single chromatographic peak with a retention time of 14.73 minutes (peak 2 in Fig.4). The amount of ImCOOH in the photooxidized UCA sample was gradually increased upon storage at -20° C.

Synthesis of imidazole-4-carboxaldehyde

(4-formylimidazole; FW = 134.5) from 4-(hydroxymethyl)imidazole-HCl.

538 mg starting material (4 mmol) was dissolved in 4 ml methanol and 500 mg $NaHCO_3$ (6 mmol) was added. The tube

was occasionally stirred for 60 min, alternatively at 4° C and at warm water temperature. CO_2 was allowed to escape from the glass tube. The mix was divided across several Eppendorf tubes and subjected to Speedvac treatment for 1 hour. Residues were white solids with light-yellow sirupy liquids. Chloroform/methanol mix 1:1 was added to the tubes with subsequent gentle warming and stirring. $NaHCO_3$ was separated by centrifugation of the combined fractions at 3500 rpm for 5 min. Clear supernatant was kept overnight at -20° C to allow the precipitation of additional $NaHCO_3$. Then, the solution was cleared by filtration and evaporated to dryness with a Rotavapor device. The residue was taken up in 20 ml dioxane with magnetic stirring and 4.4 mg MnO_2 (activated; for synthesis) was added in the same flask. The residue may not have been dissolved completely in first instantion. The mix was refluxed for 2 hours on a paraffin oil bath. The warm solution was filtered and MnO_2 was washed once with warm dioxane. Dioxane was evaporated with the Rotavapor® yielding a white and yellow fine cristalline solid. Crystallization was carried out in methanol repeated times. Small volumes of methanol were required, because the residue dissolved well in methanol.

Yield: ~ 20 mg (lit: ~ 475 mg) of fine off-white crystals.

M.p.: 167 - 168° C (lit: 173 - 175° C)

M.p.: 4-(hydroxymethyl)imidazole-HCl :108 - 111° C

M.p.: imidazole-4-carboxylic acid : 294 - 295° C

(lit.: Battersby AR et al., J Chem Soc (Perkin I) 43 - 51, 1980)

The results show that similar sets of several UCA oxidation products can be formed with UV irradiation and without (Fenton reaction type). Three products were

identified so far. We assume that these compounds occur in the upper layer of the epidermis as well and a method will be developed to determine UCA oxidation products *in vivo*. The simultaneous break-down of ImCHO and the gain of ImCOOH after photooxidation has led to our speculation that ImCHO is slowly oxidized to ImCOOH during storage. Many aldehydes are gradually oxidized to the corresponding carboxylic acids in contact with oxygen species.

Two phenomena out of the puzzling mechanism of *cis*-UCA induced immuno-suppression can be solved if UCA oxidation products would have immunosuppressive properties. First, the abrogation of the immunosuppression by antioxidants (19-21) in the model of contact hyper-sensitivity measuring ear swelling response. In our scope, the formation of UCA oxidation products is prevented, because of neutralization of the hydroxyl radicals by the antioxidants. Second, the lack of correlation between *cis*-UCA formation by UV-B and UV-A (18). No immunosuppression was found with UV-A irradiaton, despite the fact that *cis*-UCA was formed. In our scope, this finding may be explained as the inability of UV-A to photooxidize UCA. Consequently, no UCA photooxidation products are formed with UV-A (results section) and because of that immunosuppression would not occur. Our findings and the above assumptions may point to a important role for UCA (photo)oxidation products in the skin immune system.

LEGENDS to FIGURES.

Figure 1. Compounds tested in this study for hydroxyl radical scavenging ability. (a) *trans* UCA, (b) *cis*-UCA, (c) L-histidine, (d) dihydroUCA or 3-(imidazol-4-yl)propionic acid, (e) imidazole acetic acid, (f) 2-methylimidazole, (g) imidazole, (h) L alanine, (i) *trans*-2-furylacrylic acid and (j) uric acid.

Figure 2. A determination of the second order rate constants of *trans*-UCA and *cis*-UCA with hydroxyl radicals. The rate constant was derived from the slope of the line (k = slope x k_{dR} x [dR] x A_0), where A_0 is the absorbance, measured in the absence of hydroxyl radical scavenger. K_{dR} was taken as $3.1 \times 10^9\ M^{-1}.s^{-1}$, derived from pulse radiolysis studies [8], and [dR] = 3 mM. The rate constants in this particular set were 8.49 and $7.33 \times 10^9\ M^{-1}.s^{-1}$ for *trans*-UCA and *cis*-UCA, respectively. The other scavengers were studied similarly.

Figure 3. Chromatograms of 80 µM *trans*-urocanic acid in 20 mM phoshate buffer pH 7.2. The initial concentration of hydrogen peroxide was 500 µM. Injection volume was 80 µl.
a. with hydrogen peroxide; not irradiated, b. without hydrogen peroxide; irradiated with a WG280 filtered xenon-arc lamp, c. with hydrogen peroxide and irradiated as 1b, d. with hydrogen peroxide and irradiated with a WG335 filtered xenon-arc lamp. Peaks assigned with A - H correspond with photooxidation products. Separation was performed on a Alltima C_{18} column with UV detection at 210 nm. The eluent consisted of 10 mM sodium phosphate pH 7.2 with 1.0 mM tetrabutylammonium hydrogen sulphate. Further experimental conditions are described in the text.

Legends (cont.)

Figure 4. Comparable chromatographic patterns in the formation of UCA oxidation products from 80 μM trans-UCA and 500 μM hydrogen peroxide in water (no buffer). Left: after Fenton oxidation with 250 μM Fe^{2+} and right:. after photooxidation with 'full' UV, containing a UV-B dose of 32 $kJ.m^{-2}$. The cis-UCA peak is missing after Fenton oxidation, due to the absence of photoisomerization. Peak assignation (A - G) was done as in Figure 1c. Peaks B,C and D refer to imidazole-1-carboxaldehyde, imidazole-4-acetic acid and imidazole-4-carboxylic acid, respectively. Chromatographic conditions were identical to those applied in Figure 3.

Figure 5. Inhibition of contact hypersensitivity as a reduction of ear swelling response from BALB/c mice. The positive control (no inhibition) was normalized to 100 %. Im-mix is a mix of the three identified imidazoles (see identifications) and POmix III is a mix of the three identified imidazoles among several other unidentified UCA oxidation products, obtained upon extensive photooxidation. Rudimental *trans*- and *cis*-UCA are present in lower amounts than 3 % (by weight).

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TABLE 1.
THE HYDROXYL RADICAL SCAVENGING ABILITY OF UROCANIC ACID ISOMERS
AND RELATED COMPOUNDS.

HYDROXYL RADICAL SCAVENGER	SECOND ORDER RATE CONSTANT $\times 10^9$ $M^{-1}.s^{-1}$	S.D.	n[b]	INHIBITION of DEOXYRIBOSE DEGRADATION [SCAVENGER]=[DEOXYRIBOSE]=3Mm %
IMIDAZOLES				
trans-Urocanic acid	8.0	0.9	8	67
cis-Urocanic acid	7.1	0.6	6	64
L-Histidine	2.6[c]	0.9	4	34
Dihydrourocanic acid	3.7	0.9	3	34
Imidazole-4acetic acid	2.2	0.1	3	30
2-Methylimidazole	11.7	2.6	5	76
OTHER COMPROUNDS				
L-Alanine	0.1	0.0	3	2
trans-2-Furylacylic acid [a]	< 0.1	-	3	<2
Uric acid	27.8	3.0	4	91

a. trans-2-furylacrylic acid was not tested in concentrations > 8mM because of poor solubility.
b. n represents the number of slopes from which the rate was calculated.
c. 2.3-3.0 x 10^9 $M^{-1}.s^{-1}$ in literature [22]

TABLE 2.

UROCANIC ACID (UCA) ISOMERS [1] after PHOTOOXIDATION

UV RADIATION SOURCE	SPECTRAL CHARACTERISTICS		DOSE kJ.m^{-2} UV-B UV-A	UCA LEFT OVER % (± SD) [2]	YIELD OF PHOTOOXIDATION PRODUCTS A.U. [3] (± S.D.) [2]	PHOTOISOMERIZATION [4] trans-UCA % (± SD) [2]	cis-UCA %
Xe arc	WG280	270 - 400 nm	37 70	43 (± 11)	347 (± 58)	41 (± 2)	59
	UV-C,-B,-A included						
Xe arc	WG305	292 - 400 nm	18 70	64 (± 6)	219 (± 14)	47 (± 3)	53
	UV-B,-A included						
Xe arc	WG335	320 - 400 nm	0 65	95 (± 5)	45 (± 8)	60 (± 2)	40
	only UV-A included						
TL12 [5]	unfiltered	280 - 366 nm	3.6 4.5	90 (± 20)	149 (± 51)	41 (± 4)	59
TL10R [5]	unfiltered	320 - 440 nm	0 324	99 (± 3)	16 (± 5)	84 (± 7)	16

[1] Initial concentration of *trans*-UCA or *cis*-UCA is 40 µM and that of hydrogen peroxide 500 µM

[2] Standard Deviation (S.D.) of duplicate measurements.

[3] A.U.: Arbitrary Units derived from peak area integration. The peaks of 8 major products were summed.

[4] This listing only applies to *trans*-urocanic acid as starting material.

[5] Philips' fluorescent tubes. Different spectral distribution and radiometric measurements as compared to xenon-arc.

TABLE 3.

TRANS-UROCANIC ACID [1] after FENTON OXIDATION

$[Fe^{2+}]$ [2] (μM)	*TRANS*-UROCANIC ACID LEFT OVER % (± S.D.) [5]		YIELD of FENTON OXIDATION PRODUCTS A.U. [4] (± S.D.) [5]	
	in phosphate buffer [3]	in water	in phosphate buffer [3]	in water
0	100 (± 1)	100 (± 3)	< 10	< 10
50	97 (± 1)	77 (± 11)	< 10	194 (± 34)
100	94 (± 5)	48 (± 7)	27 (± 5)	272 (± 6)
250	83 (± 3)	19 (± 8)	36 (± 3)	423 (± 76)
500	78 (± 12)	< 4	49 (± 9)	511 (± 35)

[1] Initial *trans*-UCA concentration: 40 µM.

[2] Fe^{2+} added before hydrogen peroxide.

[3] 10 mM sodium phosphate buffer, pH 7.2

[4] A.U.: Arbitrary Units derived from peak area integration. The peaks of 8 major products were summed.

[5] Standard Deviation (S.D.) of duplicate measurements.